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December 14, 1976

Dr. Benjamin Lewin
Cell
The MIT Press
28 Carleton Street
Cambridge, Massachusetts 02142

Re: D1036

Dear Benjamin:

Many thanks for sending your reviewers' comments about the enclosed manuscript by Padgett et al. In general I agree that the reviews were fair and constructive, and I appreciate the care with which the manuscript was considered. I feel that the alterations and additions prompted by the comments have improved the manuscript in several places. Let me respond in order to these comments.

The first reviewer (the one who begins "through physical separation...") opens by commenting that the method we have used here may soon "be superceded in resolution and certainty by other methods," presumably by restriction endonuclease analysis. Since our lab is probably the furthest along in the analysis of RNA tumor virus-specific DNA by these other methods (we have already, for example, looked at restricted DNA from several lines of uninfected chickens and have a partial restriction map of the ASV genome), I feel relatively confident that it will be at least a year before we will be able to confirm that RAV-0 and "sarc" DNA are unlinked using this method. Moreover, even when they can be shown to be unlinked by the newer methods, the issue of whether they are on the same linkage group (i.e., chromosome) will not be approachable by this method or by genetic analysis. So I don't think you should conclude that the experiments in this paper will soon be thought outmoded. The only other evidence bearing on the linkage of RAV-0 and "sarc" sequences is not direct-the size and regulation of RAV-0 and "sarc" RNA in normal chicken embryos; that evidence is already discussed in the manuscript and will soon (it turns out) be submitted to Cell.

The second issue raised by this reviewer concerns our evidence that the MSB-1 cell is not infected by horizontally-transmitted avian leukosis or sarcoma viruses. He acknowledges that we "provide strong evidence against productive infection" but he is concerned about "non-productive" infection, and in particular about a non-productive infection which causes such a small increment in number of viral DNA copies that we would not detect it by C_0t analysis and which does not lead to detectable expression of viral genes. I agree that it is impossible to exclude the possibility that infection has added a single, perhaps incomplete, copy of viral DNA which is not expressed. However, the possibility seems very remote, since we have shown these cells to be permissive for viral replication by at

least three assays and since there is no precedent for the notion that the viral structural antigen would be absent from infected permissive or non-permissive cells, even when infected by defective viruses which make noninfectious or no particles.

Most importantly, however, the reviewer's claim that "exogenous infection by an avian oncornavirus would affect the principal conclusions of this manuscript" is incorrect. The principal point of the paper is that the microchromosomes contain "sarc" DNA but not RAV-0 related DNA; therefore, if by some very remote chance the cells were abortively infected, the added DNA must be in the macrochromosomes and cannot have any effect upon our conclusions.

I do agree with the reviewer that the C_0t curve (former Figure 3) cannot, of itself, exclude exogenous infection, and I think that this experiment was given undue emphasis in the first version of the paper. In retrospect, I really don't feel that it serves much purpose; it not only consumes the reader's time and diverts his purpose, but also doesn't establish much more than that MSB-1 DNA looks like chicken embryo DNA by C_0t analysis. As a result, I have deleted the Figure and expanded my description of the other, more sensitive assays we have used to moniter infection, putting a description of the C_0t analysis into a more suitable setting. Basically, I think it is a waste of paper to describe in detail all the moniters of infection used here since the methods are not novel and have been described in the cited literature. If you do not agree, I would be willing to expand the manuscript to include all the negative data. For your interest, I have enclosed a copy of the radioimmunoassay.

Although the relevant figure has been removed, I should still like to respond to the comments about it. (1) Although the number of ^{14}C cpm were relatively few to simplify counting in the ^{3}H channel, the $C_0t_{1/2}$ is the expected value for chicken unique sequence DNA and was validated on other occasions with the same cellular DNA. (2) Uninfected chicken embryo cells have been examined with the same method and yielded precisely the same results. The manuscript now makes this point and provides the appropriate reference to our earlier work. (3) The MSB-1 DNA was extracted from whole cells by a procedure similar to that used for the DNA from the chromosomal gradient. Since we do not see an increment in copy number in the C_0t curve, I do not understand why the reviewer raises the possibility of unintegrated DNA.

The reviewer mentions a paper (well-known to me) by Peters et al. in the context of questioning whether the MSB-1 cells might be infected. The paper cited is, to my mind, of little value by current (if not past) standards, asking the reader to believe, for example, differences between 1% and 2% annealing with viral cDNA. Moreover, there is no effort made to distinguish between endogenous and exogenous viral RNA in the studied cells and hence no respectable evidence for infection.

The reviewer then raises several perfectly legitimate points about the annealing results. These questions arose principally because we failed to provide adequate information about the hybridization assay or sufficient detail about each experiment. By an expansion of the experimental methods section on the assay and by providing more precise information about the annealings shown in Figures 5 and 6 (in the revised version), I think these difficulties have been ameliorated. In particular, I have provided an explanation for the reduced annealing with $cDNA_{B77}$

seen in Figure 6 and have clearly indicated the amount of DNA in each point. In addition, the number of cpm of each hybridization reagent used and the specific activities of the reagents have been added.

The second reviewer raises an important issue in point (1), an issue that we had previously alluded to in part, in a vague statement to which he rightfully objected in point (5). We have now removed the unclear sentence at the end of p. 7 and have inserted a paragraph which contains the appropriate qualifications and the supporting references. In fact, I have gone a step beyond the reviewer and added some qualifications he does not raise (these are related to poorly characterized viruses which have been induced from normal chicken cells).

(2) The possibility of macrochromosomal breakage was, I believe, adequately mentioned both in the results and discussion sections of the first version. The reviewer's comment does not appear to call for a response.

(3) I have considered the issues raised here in contending with the comments of the first reviewer (his "second issue"). The same response applies here. Moreover, I do not see how presentation of data with infected cell DNA would help the manuscript; we and many others have already shown that infected cells have increments in copy number detectable by C_0t analysis; but no number of "positive controls" will silence the skeptic who says that the increment in MSB-1 is simply too small to see. I prefer to emphasize the argument (made above and in the Discussion) that even this remote possibility of undetected and unexpressed DNA would not affect any conclusions we have drawn in this study.

(4) Certainly there are no new conclusions to be drawn from the data shown in the last figure; however, I think it is important to retain it. The first gradient shown (now Figure 5) contained considerably more DNA and permitted analysis with several hybridization reagents; hence we must show it. But to provide an adequate account of the methodological difficulties, we must also describe the contamination of gradient fractions with intact nuclei. The reader will then (quite sensibly in view of the incompleteness of the chromosomal fractionation) wish to see results of a gradient free of this complication. Such results are shown in the final figure; in fact, the fractionation appears cleaner, although the extent of annealing appears relatively low. As noted in response to the first reviewer, this problem has now been explained in the text. (There was less DNA per annealing reaction, and the second preparation of ^{32}P $cDNA_{B77}$ generally annealed less efficiently, as demonstrated in the accompanying control annealings with unfractionated MSB-1 DNA.)

The reviewer raises some questions about C_0t values and cellular DNA excess in his discussion of the analysis of gradient fractions; his comments suggest that he has not fully understood the assay, probably as a consequence of the very paltry explanation of it originally provided. The assay is based upon the simple observation that under standard conditions of annealing (salt, incubation time, volume, and amount of cDNA) relatively linear increases in annealing of cDNA (up to about 40%) are observed with increasing amounts of unlabeled homologous DNA in the test samples. The procedure makes possible analysis of rare DNA sequences in gradients and gels, conditions under which vast DNA excess and complete C_0t curves for each fraction would clearly be impossible. Moreover, the results can be normalized by control annealings with suitable standards. (I enclose a recent J. Mol. Biol. paper from our lab which illustrates the convenience and accuracy of the technique. Moreover, the assay has been widely used in other labs to measure viral and cellular genes,

and it has been defended on pragmatic and theoretical grounds by J.O. Bishop, among others.) Thus the C_ot values and extent of DNA excess are not relevant and do not need explicit statement, although they could be computed from the information provided. What is more to the point, the Experimental Methods section now provides a more substantial account of the annealing assay and additional references to its use.

I hope that my comments and revisions will prove satisfactory and that this paper can be accepted for publication in Cell. To ease your assessment of the changes, I have sent you both the new and old versions and have marked in light pencil the regions in which substantive change has occurred.

With best regards,

Harold

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HEV:bc